



Screening and Isolation of Extracellular Protease Producing Bacteria from the Maharloo Salt Lake

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Abstract

Screening and identification of moderately halophilic protease producing bacteria from different regions of Maharloo, a hypersaline lake in the southern area of Iran, were the objectives of this study. In the preliminary screening, 16 isolates exhibited proteolytic activity on saline skim milk agar. All isolates were able to grow comfortably in the media containing 7-15% of salt. Protease activity was assessed through *in vitro* colorimetric assays for general proteases and the strains were identified by 16S rDNA molecular marker. In comparison to Gram-negative bacteria, the Gram-positive rods, displayed higher proteolytic activities, and *Bacillus* sp. BCCS 041 was found as the highest protease producing strain with 0.43 U/ml of supernatant activity.

Keywords: *Bacillus* sp; Moderately halophilic; Protease.

Received: February 18, 2011; **Accepted:** April 27, 2011

1. Introduction

Proteases (EC.3.4) are a distinct subgroup of hydrolytic enzymes which catalyze the cleavage of peptide bonds in proteinous substrates. Depending on their mode of action and catalytic mechanism, proteases are divided into four major groups including: serine protease (EC. 3.4.21), cysteine (thiol) protease (EC. 3.4.22), aspartic proteases (EC. 3.4.23) and metallo-protease (EC. 3.4.24) [1]. In addition to their pivotal metabolic and physiological importance, they possess diverse

commercial applications worldwide [1]. Their applications are vast in detergents, chemicals, food, pharmaceutical and leather tanning industries. Most commercial proteases belong to the neutral and alkaline proteases which are produced by microorganisms in particular *Bacillus* genus. Other promising applications have been associated in potential biotechnological processes and waste water treatment [2, 3].

Since the majority of industrial processes are accomplished under harsh conditions, it would be of great importance to enjoy microbial enzymes that demonstrate optimal activities at wide ranges of pH, temperature and salt concentration. Microorganisms inhabiting in hypersaline environments are a

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remarkable source for producing such enzymes. They are expected to have specific proteins presenting characters which are different from proteins produced by organisms from nonsaline environments. They may have the capability to produce halophilic enzymes which have potential application in situations that require tolerance of high salt concentration. Moderately halophilic bacteria are a group of halophilic microorganisms which can grow optimally in media containing a wide range of salt concentrations (3-15% NaCl) [4]. They are phylogenetically very diverse and show important advantages for being used as a source of halophilic enzymes [5, 6]. The extensive biochemical variety and ease of genetically manipulation of these enzymes could generate new ones for various biotechnological applications [6-8]. Hypersaline lakes, with high salt concentration which is near saturation, are extreme environments; yet, they are the habitats of remarkable microbial communications. Hence, they are regarded as productive biological ecosystems. These microorganisms use different strategies for preserving their cell structure and function in highly saline conditions. They may produce compounds of industrial interest, such as extracellular hydrolytic enzymes with diverse potential applications in the industries [7]. Therefore, conducting researches for isolation of moderately halophiles able to produce extracellular enzymes are of great importance. In this study, we describe the screening of extracellular protease producer halophilic bacteria, isolated from Maharloo hypersaline lake located in the south of Shiraz, Iran. The salt concentration of this lake fluctuates periodically. It shows lower salinity at wet seasons and higher salt concentrations during dry seasons, therefore, it may contain moderately halophilic and halophilic bacteria.

2. Materials and Methods

2.1. Isolation and screening of moderately

halophilic bacteria for proteolytic activities

Sediment and water samples were collected from different parts of Moharloo salt lake in the south of Shiraz, Iran. All samples were serially diluted with sterile distilled water and the dilutions were streaked on saline nutrient agar plates (containing 7% NaCl). After 24 h of incubation at 37 °C, single colonies were screened for proteolytic activity in a saline skim milk agar medium that contained 2% (w/v) skim-milk, 1% (w/v) tryptone and 7% (w/v) NaCl [6]. The clear zones of hydrolysis over the next 24 h were taken as evidence of proteolytic activity, qualitatively [2].

2.2. Protease production

The protease producing isolates were inoculated in 250 ml Erlenmeyer flasks containing 50 ml saline skim-milk broth and incubated at 37 °C and 150 rpm [9]. After incubation for 48 h, the cultures were centrifuged at 4500×g for 10 min at 4 °C, and the cell free supernatants used as crude enzyme for extracellular protease assay.

2.3. Protease assay

Protease activity was determined using casein as a substrate. The assay mixture contained 1.1 ml of 1% (w/v) casein in 0.1 M Tris-HCl buffer (pH 8.0) and 0.1 ml of enzyme solution. After incubation at 37 °C for 30 min, the reaction was terminated by adding 1.8 ml of 5% (w/v) trichloroacetic acid. Then each test tube was centrifuged at 4000×g for 20 min and the absorbance of the supernatant was determined at 280 nm. One international unit (IU) of protease activity is the amount of enzyme which liberates 1 μmol of tyrosine per min [3, 11].

2.4. Identification of bacteria

Initial morphological identification of isolates was done by Gram staining. Further characterization was done on the basis of 16S rDNA gene sequencing. Different

Table 1. Protease activity (U/ml supernatant) and lengths and specific accession numbers of the 16S rRNA region of the isolated halophilic bacteria.

Halophilic bacteriai	Accession number	Length (base pair)	Activity (U/ml)
Bacillus thuringiensis BCCS 038	FJ624483	804	0.18
Paenibacillus sp. BCCS 042	FJ645733	776	0.19
Bacillus sp. BCCS 041	FJ645731	824	0.43
Bacillus sp. BCCS 040	FJ645730	787	0.17
Aeromonas sp. BCCS 037	FJ619745	802	0.39
Bacillus sp. BCCS 043	FJ645732	811	0.20
Halobacterium sp. BCCS 039	FJ645729	792	0.18
Halobacterium sp. BCCS 030	FJ529204	821	0.09
Aeromonas veroni BCCS 025	FJ429320	720	0.07
Bacillus sp. BCCS 034	FJ607328	802	0.22
Bacillus sp. BCCS 032	FJ605152	880	0.01
Bacillus sp. BCCS 036	FJ619744	802	0.10
Bacillus subtilis BCCS 027	FJ517538	832	0.07
Bacillus subtilis BCCS 033	FJ607327	796	0.01
Bacillus sp. BCCS 031	FJ554670	809	0.18
Bacillus subtilis BCCS 028	FJ517539	784	0.17

morphological and cultural characteristics of the protease producing isolates were studied compared with standard description of Bergey's Manual of Determinative Bacteriology [9].

2.5. PCR amplification of the 16S rDNA and sequence determination

The purified protease producing bacterial isolates were grown in saline nutrient broth containing 7% (w/v) NaCl. After centrifugation at 4500×g for 10 min at 4 °C, and twice washing, the pellets were selected for chromosomal DNA extraction and PCR amplification. Bacterial DNA was extracted using heat extraction method. The 16S rRNA gene was amplified by PCR using the universal prokaryotic primers 5'-ACGGGCGGTGTG-TAC -3' and 5'-CAGCCGCGTAATAC-3'. PCR was performed in a final volume of 50 µl containing PCR amplification buffer (1X), Taq DNA polymerase (2.5 U), dNTPs (4 mM), primers (0.4 µM) and template DNA (4 ng). Amplification conditions were as follows: initial denaturation at 94 °C for 5 min., 10 cycles at 94 °C for 30 s, 50 °C for 30s and 72 °C for 2 min., 20 cycles at 92 °C for 30 s, 50 °C for 30 s, and 72 °C for 2.5 min. with a final

extension of 72 °C for 5 min. Taq polymerase was added to the reaction after the first denaturation step. The lower denaturation temperature (92 °C) during the 20 cycle steps would prevent loss of enzyme activity [11]. The samples were electrophoresed in a 1% (w/v) agarose gel containing ethidium bromide (1 µg/ml). A single ~800 bp of DNA fragment was extracted from the gel using a Core Bio Gel Extraction Kit. The sequence was determined by the CinnaGen Company. The data were submitted at GeneBank database. The DNA partial sequences were aligned and compared using BLAST algorithm for finding homologous sequences in the GeneBank database in the National Centre for Biotechnology Information (NCBI). A bioinformatic tool, GeneDoc software, version 2.6.002 was used for more investigation of sequence alignments in the most protease producing *Bacillus* sp.

3. Results

3.1. Isolation and screening of protease producing bacteria

Among 40 isolated bacteria, 16 isolates (40 %) were protease producer and clear zone around colonies was considered as the

evidence of protease production. Since there is not necessarily good correlation between zones of clearing around colonies on skim-milk agar plates and levels of protease activity, all 16 isolates were identified and cultivated in broth medium and the extracellular protease activity was assessed.

3.2. 16S rDNA sequence analysis

Initial morphological identification showed 12 isolates were gram-positive, spore forming rod-shaped and four of them were gram-negative. The PCR amplification of 16S rDNA gene revealed a single band of amplified DNA product of ~800-bp, indicating efficient amplification. The DNA sequences were published in the NCBI databases under the specific accession numbers. The amount

of protease production (U/ml of supernatant) and the lengths of the 16S rDNA region of the strains and their specific accession numbers are listed in Table 1. The result of PCR blasted with other bacterial sequences in NCBI. Edited sequences were used as queries in BLASTN searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). From 16 protease positive isolates, 11 strains (68.75 %) were identified as *Bacillus* species and *Bacillus* sp. BCCS 041 had the highest proteolytic activity (0.43 U/ml supernatant). The result of the alignment by GeneDoc software was shown in Figure 1. A total of 824 nucleotides of the partial sequence of *Bacillus* sp. BCCS 041 were 99 % similar to the 16S rRNA genes in recorded strains of *Bacillus subtilis* in NCBI (Figure 1).

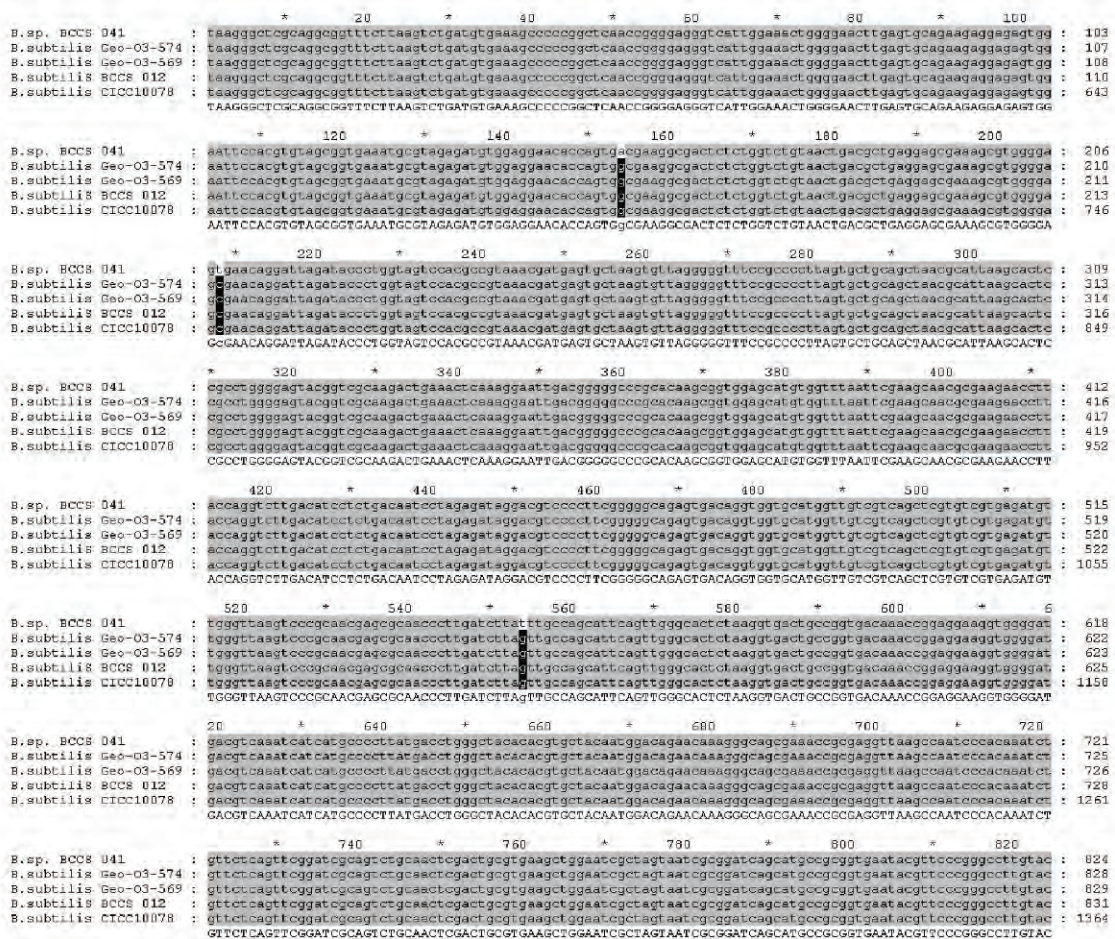


Figure 1. 16S rRNA gene sequence investigation of *Bacillus* sp. BCCS 041 by bioinformatic tool, GeneDoc software version 2.6.0.02.

4. Discussion

Proteolytic bacteria are widespread in nature and are able to grow under various growth conditions, such as different temperatures, pH and ionic strength. The presence of halotolerant protease in the halophilic bacteria could be applied in industrial processes where the concentrated salt solution used would inhibit ordinary proteases [2, 3, 7]. In this experiment, 16 bacterial isolates were able to produce proteolytic enzymes. Of these, 12 (75%) strains were Gram positive, including *Bacillus* spp. and *Paenibacillus* spp., 2 (12.5 %) strains were *Halobacterium* spp., which were Gram negative, and 2 (12.5%) were archaea, *Aeromonas* spp. In a similar study, Rohban and his associates [7] isolated 231 moderate halophilic and 49 extremely halophilic bacteria from Howz Soltan Lake. Among which there were 172 (61.4%) Gram-positive rods, 56 (20%) Gram-negative rods and 52 (18.6%) Gram-positive cocci. They found that 70 strains of Gram-positive rods, 13 strains of Gram-positive cocci and 17 strains of Gram-negative rods were protease producer. These data are in agreement with our findings and revealed that Gram positive bacteria are the dominant proteolytic species in these saline environments. Aerial distribution of the dormant spores probably explains the occurrence of *Bacillus* in most habitats. Ghasemi and his collaborates [12] isolated 9 *Bacillus* strains of 13 halotolerant amylase producing bacteria from soil and water samples of Maharloo hypersaline lake in the south of Shiraz, Iran. In addition Olajugbe and Ajele [13] have isolated 25 bacteria from soil, of which nine isolates were identified as *Bacillus*. They were *Bacillus brevis*, *B. licheniformis*, *B. subtilis*, *B. macerans*, *B. mycoides*, *B. coagulans*, *B. polymyxa*, *B. cereus* and *B. megaterium* species. These findings confirm that *Bacillus* species are widespread in the most extreme environments. The genus *Bacillus* is well

known as an enzyme producer and many industrial processes use species belonging to this genus for commercial production of enzymes [2]. Members of this genus are used for the synthesis of a very wide range of important medical, agricultural, pharmaceutical and other industrial products. These include a variety of antibiotics, enzymes, amino acids and sugars. The results of this work indicate that the moderately halophilic bacterium, *Bacillus* sp. BCCS 041, could display a potential role in protease production in biotechnological processes.

Acknowledgements

This work was supported by a grant from the Research Council of Shiraz University of Medical Sciences, Shiraz, Iran.

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